Improved procedure for high quality RNA extraction from dust in the indoor environment

Undergraduate Thesis

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Abstract

We spend 90% of our time indoors where we are exposed to microbes and chemicals present in house dust. Some of these harmful compounds include allergens and mycotoxins produced by fungi. These secondary metabolic products are known to have significant, negative effects on human health. However, we do not yet know how changes in environmental conditions can alter gene expression and microbial production of these harmful compounds. Microbial function in dust is difficult to measure because of the high concentration of RNAses, which quickly degrades RNA. RNA extraction is also difficult because the lack of stability in its structure. The purpose of this study was to develop a high quality RNA extraction procedure from house dust for use in the determination of gene expression related to allergen production. As a case study, we considered how different levels of water activity influence gene expression of the common fungal allergen Alt a 1. Water activity levels included 0.5, 0.85, and 1.0 a_w, and the indoor setting was simulated by incubating dust samples in carpet pieces in incubators. RNA extraction was performed using Qiagen RNeasy PowerMicrobiome extraction kits. After completing cDNA synthesis and qPCR analysis, we measured expression of allergen Alt a 1 in samples that had been spiked with *Alternaria alternata.* Modifications to an existing RNA protocol (Qiagen) intended for fecal samples improved the RIN^e score from below detection to 7.5. We confirmed production of Alt a 1 in all three samples incubated at 1.0 a_w, 1 of the 3 samples incubated at 0.85 a_w, and none of the samples incubated at 0.50 a_w. Overall, this work overcame obstacles related to high-quality extraction of RNA from house dust. Future work will demonstrate important implications for moisture control in indoor environments in regards to allergen production and chronic exposure.

Acknowledgements

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1. Introduction

1.1 Asthma and exposure pathways in the indoor environment

In the United States about 25 million people suffer from asthma, with cases more than doubling since 1980 (CDC, 2011; Akinbami et al., 2012). Costs associated with asthma in the United States were around $81 billion in a 2013 study (Nurmagambetov et al., 2017). Asthma has both genetic and environmental causes. Important environmental exposures occur indoors, where we spend 90% of our time (Klepeis, 2001). Exposures such as dampness and mold in housing are associated with negative effects on human health (Fung and Hughson, 2003; Dales et al., 1991). Costs associated with mold exposure in homes amounts to around $22.4 billion dollars annually (Mudarri, 2016).

In buildings, increased moisture can result in rapid fungal growth and cause irritation in occupants (Pasanen et al., 2000; Fung and Hughson, 2003; Haleem and Karuppayil, 2012). Several studies have linked mold exposure and indoor moisture problems to acute bronchitis, allergic rhinitis, and asthma (Zock et al., 2002; Mudarri, 2016; Fisk, 2002). Development of asthma in children and infants has also been linked with exposure to damp indoor conditions (Mendell at al., 2011). Intensity of fungal allergen exposure has been linked to asthma severity and respiratory problems (Lau et al., 2000; Denning et al. 2006). This is especially concerning since we spend 90% of the time indoors (Klepeis et at., 2001). Airborne and resuspended fungi can lead to ingestion (Qian et al., 2014). In the US the average unintentional house dust daily intake is 60mg for children and 30mg for adults (Fromme et al., 2014). Indoor dust collected from floors, 97% of which were carpeted, displayed geometric means of 71,118-81,367 CFUs of total fungi per gram of dust (Jacob et al., 2002). Amongst large populations, the savings estimates from improved indoor environmental quality are 8-25%, or between $1.8 and $5.6

billion dollars (Fisk, 2002). More work is still needed to create standards for assessing fungal growth (Green et al., 2017).

1.2 Alternaria alternata and secondary metabolic products

Many fungi produce harmful secondary metabolic products such as allergens and mycotoxins that can lead to allergic disease and adversely affect human health (Mendell et al., 2011; Pieckova and Wilkins, 2004; Samet and Spengler, 2003). One of the most common airborne indoor fungi is *Alternaria spp.* (Salo et al., 2006; Thomma, 2003). In a study of *Alternaria* antigens present in U.S. homes, over 95% of samples collected had detectable levels of *Alternaria* antigens, and moisture problems were determined to be a contributor to levels (Salo et al., 2005). *Alternaria alternata* is a potent producer of allergens and common in indoor air (Gabriel et al., 2016; Hedayati et al., 2009). The constant exposure of *A. alternata* present in summer, autumn, and spring seasons, especially in the Midwestern United States, is similar to more chronic asthma causing allergens like dust mites and cat dander (Ren et al., 2001; Sanchez, 2001; O'Hollaren et al., 1991). In an urban setting, *Alternaria* allergens were found to be the second strongest risk factor for asthma, while in a rural location *Alternaria* allergens were deemed insignificant (Perzanowski et al., 1998). Changes in humidity and temperature can cause *A. alternata* to sporulate and result in increased airborne concentrations (Timmer et al., 1998; Pulimood et al., 2007).

Alt a 1 is a major allergen protein produced by *A. alternata* with a B-barrel structure unique among fungi (Sanchez, 2001; Chruszcz et al., 2012). *A. alternata* is in the order Pleosporales, and other species in this order also produce this Alt a 1 allergen (Sáenz-de-Santamaría et al., 2006). Of individuals with allergic sensitization to *A. alternata*, 80-90% have

IgE antibodies that specifically recognize Alt a 1 (Vailes et al., 2001). Exposure to this allergen has been linked to adverse respiratory symptoms in individuals sensitive to *A. alternata* and can be used to determine risk factors and severity (Brito et al., 2012).

1.3 Quantifying fungal gene expression

Quantifying gene expression is important for understanding how secondary metabolic products such as allergens and mycotoxins are produced at different water activity levels. Gene expression quantification is studied by measuring mRNA. mRNA, or messenger ribonucleic acid, is a polymer which can fit through nuclear pores and copy genes in DNA for protein creation (Wan and Chaterjee, 2018). RNA shows what genes are activated or off, as well as timing of each, and level of expression (Ozsolak and Milos, 2011). Upregulation and downregulation of a gene means an increase or decrease, respectively, of cellular components such as protein production. Methods used to measure gene expression include serial analysis of gene expression (SAGE), DNA microarray, and RNA sequencing (Robertson, 2018). SAGE is a tag based sequencing approach that provides digital gene expression levels and can be used when the transcriptome is unknown (Velculescu et al., 1995; Wang et al., 2009). This method requires a large quantity of input RNA (Datson et al. 1999), which can be a drawback depending on how much sample is available. This method requires relatively expensive technology and unknown tags can result in many false positives (Yamamoto et al., 2001; Wang et al. 2009). DNA microarrays are able to test the expression of many genes at once and have test sites that can vary in size; however, they are not always sensitive for very low or high quantities of expression (Heller, 2002; Wang et al. 2009). RNA sequencing involves cDNA synthesized from RNA as an input in sequencing libraries. This method determines relative abundance of RNA (Robertson,

2010) and can identify putative genes (Conway et al., 2014), making it ideal for organisms with unknown genomic sequences (Wang et al., 2009). RNA sequencing is considered by many to be the most ideal method of determining differential gene expression, though sequencing libraries can make normalizing data complex (Finotello and Di Camillo, 2015). After cDNA production, these specific genes of interest can also be quantified using qPCR.

High quality, purity, and concentration of extracted RNA is difficult to achieve in indoor dust samples. There has been little research done on modified RNA extraction procedure for house dust. RNA quality is often measured with RIN^e scores, obtained from Agilent 2200 TapeStation Bioanalyzer (Agilent, Santa Clara, CA, USA). RIN^e stands for RNA Integrity Number equivalent, and is a similar quality score as RIN, which is measured using a traditional bioanalyzer (Padmanaban, 2012). RIN^e scores are on a scale from 1 to 10, with 10 being high quality RNA regions with distinguishable 28S and 18S peaks and minimal degradation (Mueller et al., 2016). It is largely accepted that high quality and purity RNA, represented by high RIN^e scores, are the one of the most important factors for reliable downstream application (Imbeaud et al., 2005). A RIN^e of 7.0 or higher is typically considered high quality RNA. Though some studies have shown that RIN^e as low as 5.0 are sufficient for downstream applications (Fleige and Pfaffl, 2006). RNA is much more prone to degradation than DNA due to the presence of RNase, which rapidly digests RNA into small fragments (Felske et al., 1996; Schroeder et al., 2006). In fungi, high amounts of endogenous RNase are present making make RNA extraction difficult (Patyshakuliyeva et al., 2014; Buckingham and Flaws, 2007). While there is little research on RNase concentration in dust, most manufacturer RNA extraction procedures list ambient dust contamination as a major route of RNase, indicating high levels present. Thus, direct extraction from dust itself may be challenging. Due to their structure, RNase is difficult to

deactivate and requires strong inhibitors (Mommaerts et al., 2015). While some RNA extraction procedures have been developed for fungi in soils and fecal material, little research has been done on RNA extraction in dust within the indoor environment (Patyshakuliyeva et al., 2014; Nannipieri et al. 2003). High quality RNA is critical for downstream use on qPCR (Fleige and Pfaffl, 2006). Studies done with bovine tissues have shown that high RIN scores correlate to lower cycle threshold values when using qPCR (Fleige et al., 2006). Low RNA quality results in higher variation, and the use of reference genes does not resolve this variation (Vermeulen et al., 2011).

Epidemiological studies have indicated that there may be an association between moisture in homes and Alt a 1 production, but there is little information about specific gene expression of Alt a 1 in different moisture conditions. Along with other factors, increased water activity typically increases fungal growth, and enhanced fungal development has been linked to secondary metabolites in fungi (Miller and McMullin, 2014; Baxi et al., 2016; Calvo et al., 2002). Water activity, a_w, is a measure of water availability, with equilibrium relative humidity (ERH) corresponding to water activity converted to a percentage. Increased ERH levels have shown to lead to an upregulation in secondary metabolic genes in fungal communities in house dust using RNA sequencing on an Illumina platform (Hegarty et al.,2018). This study considered 50%, 85%, and 100% ERH and determined that fungi in dust were metabolically active at all three levels. Within this study the number of upregulated allergen contigs was nearly double at 100% ERH than 50% ERH (Hegarty et al.,2018). Contigs are continuous regions developed by overlapping gene segments between sequence reads that are used to assemble a genome (Baker, 2012). GO mapping, which identifies the roles of genes and gene products (Ashburner et al.,

2000), determined that there were 1.5 times more contigs representing secondary metabolic processes at 100% ERH than at 50% ERH (Hegarty et al., 2018).

The goal of this project is to develop a reproducible, high quality RNA extraction procedure for indoor dust for the use of determining allergen gene expression at different ERH levels. This study will focus specifically on Alt a 1 expression; however, a successful RNA extraction procedure will allow for future gene expression studies of many types of fungal products. This study has important implications for human health within the indoor environment and indoor environmental quality standards.

2. Methods and Materials

2.1 Sample collection and experimental set-up

Dust samples used for RNA extraction were collected from two sites. Site 1 dust was collected in May 2017, and site 2 dust in March 2019. Both sites were located in Columbus, Ohio, and vacuum bags were taken from each. A 300µm sieve was used with sterile tin foil to remove dust from other debris in the vacuum bag. The Institutional Review Board at Ohio State University approved this study.

In order to simulate the indoor environment, the indoor dust was embedded into 10.75 cm x 10.75 cm carpet squares. Carpet squares were collected from the same home as the dust in site 1 and used for both site dusts. Following a modified ASTM method, 250mg of dust was embedded into carpet samples (ASTM, 2013; Dannemiller et al., 2016). The samples were incubated for one week at 25^{o}C in parafilm sealed, sterile glass containers under differing ERH levels to model damp, indoor environments. Specifically, the humidity was regulated with sodium chloride or magnesium chloride salt solutions placed in the chamber at either 50%, 85%, 100% ERH. During trials of method development all samples were incubated at 100% ERH.

Samples under differing ERH levels and trials were conducted using site one dust. Water activity of salt solutions was determined using the AquaLab 4TE (METER Group, Pullman, WA, USA) water activity meter (Meter Food, USA). Onset HOBO loggers were used to record the internal temperature and relative humidity of the jar (Onset, Bourne, MA, USA). Dust was extracted through 19mm x 90mm Whatmann thimbles using a Eureka "Mighty Mite" vacuum. All samples were done in triplicate. Site two dust was tested towards the end of the study to determine if dust composition would affect the quality output using the modified procedure.

Table 1. *Product types used and descriptions*

Product	**Process**	**Manufacturer**
RNeasy PowerMicrobiome Kit	RNA extraction	Qiagen
RevertAid First Strand cDNA Synthesis Kit	cDNA synthesis	Thermo Scientific

2.2 RNA extraction procedure using phenol based lysis

The dust was removed from the carpet squares after the one week incubation. RNA was extracted using Qiagen RNeasy PowerMicrobiome extraction kits (Qiagen, Hilden, Germany) (Table 1). RNeasy PowerMicrobiome kits were designed for total RNA removal from high PCR inhibitors like stool and gut material. Kits utilize bead beating with 0.1mm glass beads, and silica spin filters for binding RNA (Qiagen, Hilden, Germany).

Since RNA can be so easily destroyed, the extraction procedure was performed immediately following dust collection to avoid damage to the RNA. Table 2 details methods used in trials for final procedure development. 100 µL of phenol chloroform isoamyl alcohol

(25:24:1) with a pH of 8.0 was added in addition to several different solutions along with 10x the amount of 2-mercaptoethanol (β-ME) (Sigma Aldrich) to isolate the DNA and RNA from the cell. An alkaline ratio was chosen in order to maximize total RNA extracted into the aqueous layer. Bead beating for cell lysis was performed in two minute intervals and samples put on ice for one minute in between intervals. Bead beating with cooling intervals have been done in similar studies as temperature has been shown to affect yield and quality (Gabriel, 2015; Leite et al., 2012). A DNase buffer was added to digest the DNA, leaving RNA to be purified (Qiagen, Hilden, Germany). Extracted RNA was stored at -80^{o}C for use in further applications.

Successful RNA extraction was defined using the Agilent 2200 TapeStation Bioanalyzer located on campus to show high-quality RNA (Agilent, Santa Clara, CA, USA). The samples were also run on the RNA setting of Nucleic Acid Quantification on the BioTek Synergy HT and Take3 plate to determine purity and concentration (BioTek, Winooski, VT, USA). An A_{260}/A_{280} ratio of 1.8-2.0 is generally considered high purity for RNA samples. Samples incubated under different humidity conditions were also run on the BioTek Synergy HT at A_{270} and A_{230} to verify no phenol or salt contamination was present.

Table 2*. Trial procedural changes and resulting RIN[e]*

Trial	Procedural Change	Resulting RIN[e]
1	x10 β-ME	Below limit of detection
2	x10 β-ME PM1 solution (Qiagen) and β-ME mixed before sample addition Reduced DNase incubation (5 minutes; originally 15 minutes)	1.6
3	x10 β-ME PM1 solution (Qiagen) and β-ME mixed before sample addition Reduced DNase incubation (2 minutes)	Below limit of detection
4	x10 β-ME PM1 solution (Qiagen) and β-ME mixed before sample addition Sample incubated at room temperature in PM1 and β-ME solution (5 minutes) Reduced DNase incubation (10 minutes)	2.4
5	x10 β-ME PM1 solution (Qiagen) and β-ME mixed before sample addition Sample incubated at room temperature in PM1 and β-ME solution (5 minutes) 70% ethanol for filter binding (instead of 100%) Reduced DNase incubation (10 minutes)	3.9
6	x10 β-ME PM1 solution (Qiagen) and β-ME mixed before sample addition Sample incubated at room temperature in PM1 and β-ME solution (5 minutes) Additional wash with PM5 solution (Qiagen) Reduced DNase incubation (10 minutes)	3.1
7	x10 β-ME PM1 solution (Qiagen) and β-ME mixed before sample addition Sample incubated at room temperature in PM1 and β-ME solution (5 minutes) Bead beating in 2 minute intervals (10 minutes total), samples put on ice 1 min between intervals	4.5

Table 2. *(continued)*

Trial	Procedural Change	Resulting RIN[e]
8	x10 β-ME PM1 solution (Qiagen) and 2-mercaptoethanol mixed before sample addition Addition of 100 µL phenol chloroform isoamyl alcohol (25:24:1) Sample incubated at room temperature in PM1 and β-ME solution (5 minutes) Bead beating in 2 minute intervals (10 minutes total), samples put on ice 1 min between intervals	7.5

2.3 cDNA synthesis

Once high quality RNA was achieved, manufacturer protocol for cDNA synthesis was performed using ThermoFisher Scientific RevertAid First Strand cDNA Synthesis Kit (ThermoFisher, Waltham, MA, USA). The oligo$(dT)_{18}$ primer was used for cDNA synthesis process. This primer was chosen over random hexamer primers in an effort to prevent degraded RNA present in samples from being synthesized (ThermoFisher, Waltham, MA, USA). Negative reverse transcription controls for all samples were made using all components of the kit except for the reverse transcription enzyme. A negative RNA control was developed using all components of the kit except for the RNA template. A positive GAPDH control RNA and gene specific primers were also supplied.

2.4 qPCR analysis and standard development

Amplification of Alt a 1 gene was tested using forward primer Alta1CF (5'-GAGGGYGACTACRTYTGGAAGAT-3') and reverse primer Alta1CR (5'-CCATGHAGCTGTTCTSGCCRCA-3') which target a highly conserved region of Alt a 1 (Gabriel et al., 2015). NCBI Primer-BLAST was used to verify primer sequences.

A reference gene was used to normalize data and reduce variation that occurs in qPCR applications due to RNA extraction, and cDNA development (Kozera et al., 2013). A reference gene was chosen based on those that were noted in similar studies as being stably expressed, regardless of experimental conditions (Thellin et al., 2019). Forward primer (5'-TCGTTGAGTAGACTCTGAATGCTG-3') and reverse primer (5'-AGCCAGATGTTCCACCCG-3') were used to detect ubcB (Llanos et al., 2015). Several studies done on reference genes in fungi have listed ubiquitin carrier protein, ubcB as a stable gene, though these studies tested stability using variations in nutritional sources rather than differing ERH (Llanos et al., 2015; Pathan et al., 2017; Kim and Yun, 2011).

Standards were created using *A. alternata*, ATCC item number 66981. 10µL were inoculated onto Potato Dextrose Agar (PDA) plates. Plates were incubated at 25ºC for two weeks. Bacterial contamination was prevented using 0.025g of chloramphenicol (Sigma Aldrich). Spores were harvested by pouring PBS-T onto plates and scraping spores from plates. The solution was added to 2mm garnet beads, shaken, and filtered. Process was repeated until 10^6 spores/µL solution was achieved.

All forward and reverse primer concentrations were run at 10µM, including the positive control primers supplied in the cDNA kit. Wells contained 25 µL total, including 2 µL of sample or standards, and 23 µL mix of forward and reverse primers, SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and nuclease free water. qPCR was run on QuantStudio 6 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The reaction cycle was initial denaturation for 15 minutes at 95ºC, followed by 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. Melting curve stage included 95ºC for 15 seconds, 60ºC for 1 minutes, and 95ºC for 15 seconds. All samples and standards were run in triplicate with Alt

a 1 and with ubcB primers. Controls were also run in triplicate and included a negative reverse transcription enzyme control for each sample, a negative RNA control, a positive RNA control, and blank controls testing master mixes.

3. Results

3.1 Modified procedure resulted in high quality RNA

All trials during method development were run on site 1 indoor dust incubated at 100% ERH. Initial trials following the standard procedure resulted in very low quality, purity, and concentration of extracted RNA. Extracted RNA was not present, or highly denatured and located around 25 nucleotide (nt) range. Procedural changes of 10x the amount of 2-mercaptoethanol, incubation before bead beating, and placing samples on ice between bead beating intervals led to RIN^e as high as 4.5. Here the 18S and 28S bands were clear, but degraded RNA was still present.

The introduction of the phenol chloroform isoamyl alcohol, along with the previous modified changes resulted in considerably higher RIN^e scores. Large 18S and 28S bands were clear and with much less degradation present. The highest RIN^e score using phenol based lysis was 7.5, which is quality RNA. Figure 1 shows the progression of the results of using modified procedures (Table 2) through bioanalyzer plots, with the last plot showing the result of the trial with the addition of phenol chloroform isoamyl alcohol.

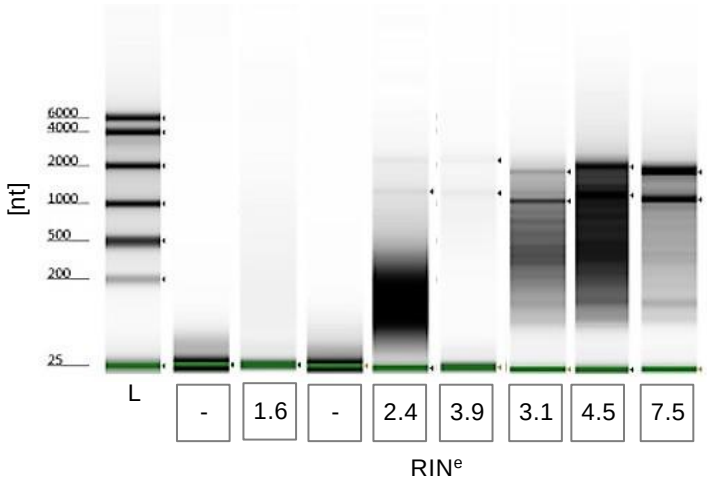

Fig. 1. *Bioanalyzer plot showing progression of RNA quality through trials.*

The procedural changes that affected RNA quality the most substantially were those occurring towards the beginning of the procedure (Figure 2). This may be due the procedural changes inactivating RNase, which is present in dust in high quantities. The success of phenol chloroform isoamyl alcohol could indicate that the dust samples contain large amounts of cellular debris or proteins that were not being removed from the aqueous layer in the original protocol.

Other procedural changes were also tested, such as spin filter binding with 70% ethanol (Table 2, Figure 1, plot with RIN^e of 3.9) in the hopes of removing degraded RNA on the membrane. While this method raised the RIN^e score from the previous trial, it resulted in a very low concentration of RNA that would make it difficult to use in downstream applications.

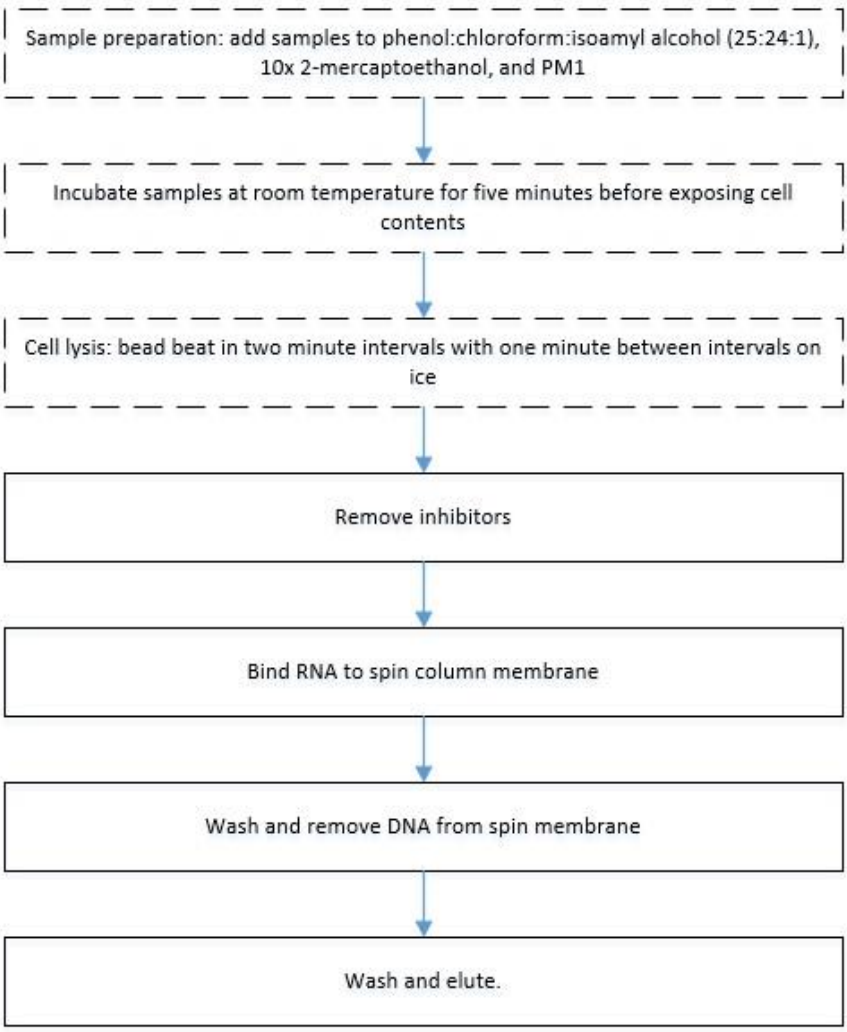

Fig. 2. *Modified procedure of RNA extraction with changed steps in dashed boxes.*

3.2 RNA quality and purity at different ERH levels

Bioanalyzer results of RNA extractions on site 1 samples run in triplicate at 100%, 85%, and 50% ERH show significantly higher quality and concentration of RNA at 100% than either 85 or 50% ERH (Figure 3). Noticeable 18S/28S peaks were seen in extracted RNA from 100% ERH. The peaks were largely absent from both 85% and 50% ERH. Since fungi is typically actively growing above 70-80% relative humidity and previous results have shown activity at all

water levels, the low concentration of extracted RNA at 85% ERH was not expected (Grant et al., 1989; Mendell et al., 2011; Hegarty et al., 2018). To determine if age of dust or site differences could impact quality, RNA from site 2 dust was also extracted using the same procedure (Figure 4). All ERH levels display noticeable 18S/28S peaks with higher RIN^e at lower ERH, though this can be attributed to the large amount of degraded RNA in the 100% ERH fast region. The quantity of extracted RNA from site 2 was higher at every ERH level than its site 1 counterpart.

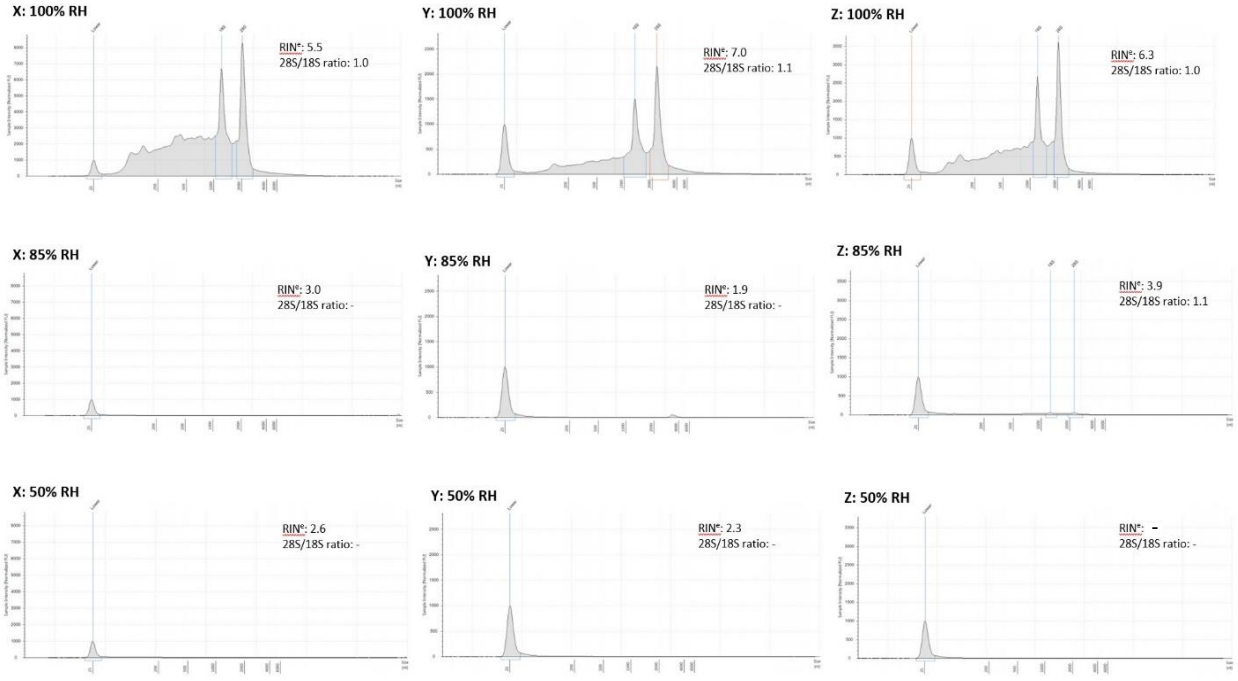

Fig. 3. *TapeStation Bioanalyzer results of Site 1 samples incubated at 100%, 85%, and 50% ERH. The y-axis or each graph indicates Sample Intensity (Normalized FU), and the x-axis indicates Size (nt)*

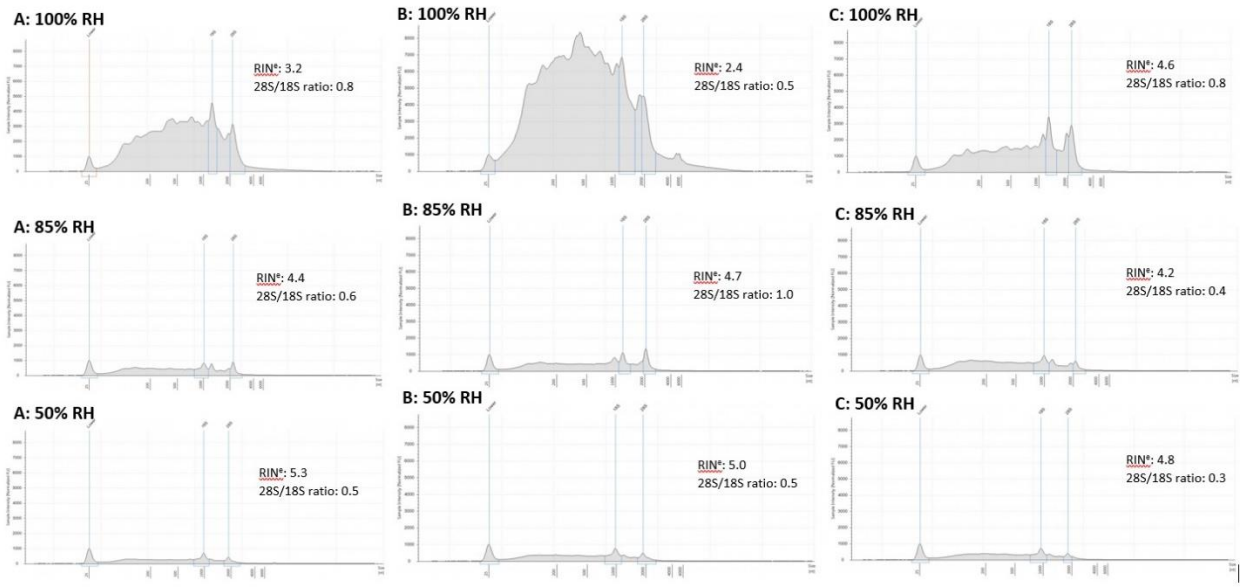

Fig. 4. *TapeStation Bioanalyzer results of Site 2 samples incubated at 100%, 85%, and 50% ERH. The y-axis or each graph indicates Sample Intensity (Normalized FU), and the x-axis indicates Size (nt)*

A_{260}/A_{280} ratio results taken from the BioTek Synergy HT showed high purity of both site 1 and site 2 samples at 100% ERH, consistently above 1.8. A_{260}/A_{280} ratios for 85% ERH and 50% ERH were low and therefore low purity. All samples had negligible values for A_{280}, representing a lack of organic and protein contamination, and negligible values for A_{270} and A_{230}, representing no phenol or salt contamination.

There was variation among each of the triplicates of the samples incubated at the same ERH (raw concentration data found in Appendix A and B). Higher ERH resulted in a lesser amount of variance in the quality of the extracted RNA in site 1 dust, though this trend was reversed in site 2 dust (Table 3). Some variation can be expected; future method testing will to be conducted to determine if variance can be reduced.

Table 3. *Coefficient of variance of quality of extracted RNA at different ERH levels*

Equilibrium Relative Humidity (%)	**RIN[e] Coefficient of Variance**	
	Site 1	Site 2
100	0.12	0.33
85	0.34	0.06
50	0.87	0.05

3.3 cDNA synthesis and qPCR show Alt a 1 present in samples

Only the site 1 extracted RNA underwent first strand cDNA synthesis. Nine samples in total were studied, with three separate trials being run at three different ERH levels. qPCR analysis showed Alt a 1 present in all samples incubated at 100% ERH, one sample incubated at 85% ERH, and none at 50% ERH (Table 4). Negative controls showed that DNA contamination was not present in any of the samples or the reagents used.

Only the most concentrated of the reference gene ubcB standards was detected, and ubcB was not detected in any of the samples. This is likely due to low concentration of ubcB forward and reverse primers. Additional testing with primer concentration and cycle settings will be done in order to determine ubcB presence. Once reference genes are detected, Alt a 1 data can be normalized to the reference and quantified.

Table 4*. Alt a 1 gene expression in different test samples*

ERH (%) / **TRIAL**	100	85	50
X			
Y			
Z			

= Alt a 1 detected in sample

4. Discussion

Initial RNA extractions from incubated dust samples resulted in poor RIN^e and were too degraded to use in downstream applications. Results of following the modified RNA extraction procedure show increases in the quality, purity, and concentration of RNA for the tested dust compared to the manufacturer's procedure. The differences in quantity and quality of extracted RNA from the different sites indicate that composition of dust may affect the quality of extracted RNA. This could affect the ability to extract RNA in studies where only small amounts of samples are available. There is little research on how dust composition changes over time, though one study testing age of dust in Danish offices found no major variations in total fungi or allergens due to dust storage (Molhave et al., 2000). However, this study only observed changes over two weeks using plating methods and did not study fungal allergens specifically. More testing is needed to determine whether modified procedures are needed for different dust ages and compositions.

Of the nine samples tested at different ERH levels, RIN^e, A_{260}/A_{280}, and concentration were all higher in samples incubated at 100% than either 85% or 50% ERH. qPCR analyses determined the presence of Alt a 1 gene in all samples at 100% ERH, one sample at 85% ERH, and none at 50% ERH. This suggests that Alt a 1 expression is influenced by relative humidity. These findings verify the finding of fungal allergens found to be upregulated in a previous study (Hegarty et al., 2018), though this study did not quantify the expression.

While there are few studies on RNA extraction in dust and allergen expression, there have been studies that show an upregulation of secondary metabolites in fungus at higher ERH levels. Putative gene expression in fungi has been linked to fungal development (Calvo et al., 2002). A study on moisture damaged building materials concluded that mycotoxins, which are secondary metabolites like allergens, were present in several samples (Taubel et al., 2011). *A.*

alternata grown in tomato medium was generally seen to produce higher levels of mycotoxins at high levels of ERH (Pose et al., 2010). Metabolic pathways related to biosynthesis of aflatoxin were also over expressed at the higher ERH level in *A. flavus*, and higher expression of gene indicators of aflatoxin, aflM and aflS were seen with higher ERH (Zhang et al., 2014; Abdel-Hadi et al., 2011). This suggests a general link that has been studied at a genus specific level between secondary metabolite production at higher moisture levels (Nielsen et al., 2004).

Increased moisture in buildings has been linked to respiratory problems, and allergic symptoms (Bornehag et al., 2001; Mendell et al., 2011). Concentrations of molds have been found at higher levels in water damaged homes of children with asthma across the United States (Vesper et al; 2006). In schools, repairs of moisture damage resulted in decreases in reported negative health symptoms (Ahman et al., 2000; Haverinen-Shaughnessy et al., 2004). These studies support conclusions that dampness and moisture may lead to asthma development and negatively affect human health (Institute of Medicine, 2000). Recommended indoor humidity is within the range of 30% to 50% to prevent mold growth (EPA, 2010), and improved moisture conditions have been reported to potentially diminish respiratory symptoms by 17%-50% (Fisk, 2002). The effects of indoor moisture levels on health are complex, but understanding them is important to revising current standards (Richardson et al., 2005).

The implications of increased relative humidity on Alt a 1 expression is still not completely understood, but findings from this study are an important step to recognizing the importance of indoor air quality. Modified indoor air standards in buildings has the potential to lead to a reduction of indoor fungal allergens, which will have a positive impact on human health. This study also goes beyond just fungal allergens, since the further refinement of this

extraction procedure will allow for the accurate quantification of a variety of gene expressions in future studies.

5. Limitations

Limitations of this study include the age of dust used within these trials and ERH study and the use of only two sites. Age of dust could be a potential factor in RNA quality and concentration. More studies need to be conducted on different dust samples. cDNA synthesis was run on samples incubated at 85% and 50% ERH, despite having low RIN[e]. Using low quality RNA in cDNA synthesis can cause degraded RNA to be reverse transcribed. The oligo$(dt)_{18}$ primer was used to mitigate this. Running qPCR with degraded RNA can increase variability (Vermeulen et al., 2011) though some studies suggest results can be used if carefully examined (Schoor et al., 2003). This study was unable to amplify all the dilutions of the reference gene, so normalization and quantification of Alt a 1 was not possible. This study also only used one reference gene, though several should be used to provide proper normalization (Derveaux, et al., 2010).

6. Future directions

Further testing will be done to study different dust samples other than the two used in this study. A procedure will continue to be developed for site 2 dust to determine if higher quality can be achieved and qPCR analysis run. The modified RNA procedure will continue to be revised to ensure reproducible, quality results. Additional steps will be tested, such as using 70% ethanol in the binding step (instead of 100%) injunction with phenol chloroform isoamyl alcohol addition, and increased filter washes. Different procedures will be run to optimize cDNA, including primer mixing with two-step RT-PCR and gene specific primers. Refined procedure

will be used to conduct further research on Alt a 1 expression using qPCR for DNA and RNA extractions. We will also explore if age of dust is associated with RNA concentration.

7. Conclusion

A reproducible RNA extraction for indoor dust was developed resulting in high quality RNA capable of being used in downstream applications. As proof-of-concept, we also confirmed production of RNA encoding for Alt a 1 in samples incubated at 100% ERH. Further research is needed to determine the quantification of expression of Alt a 1. The procedure developed in this study will allow future research to be conducted on gene expression and fungal exposure from dust in the indoor environment. This expression can result in important insights that can lead to the development of improved indoor moisture standards and benefit overall public health.

Appendices

Appendix A. Site 1 raw concentration data from Agilent 2200 TapeStation Bioanalyzer

Trial	Equilibrium Relative Humidity (%)	RIN[e]	Concentration, pg/uL
X	100	5.5	15200
Y	100	7.0	2440
Z	100	6.3	4710
X	85	3.0	118
Y	85	1.9	65
Z	85	3.9	178
X	50	2.6	170
Y	50	2.3	57.7
Z	50	-	44

Appendix B. Site 2 raw concentration data from Agilent 2200 TapeStation Bioanalyzer

Trial	Equilibrium Relative Humidity (%)	RIN[e]	Concentration, pg/uL
A	100	3.2	19100
B	100	2.4	33800
C	100	4.6	9810
A	85	4.4	3100
B	85	4.7	3600
C	85	4.2	3960
A	50	5.3	1870
B	50	5	2200
C	50	4.8	2220

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